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Research Article

ASSOCIATION OF *AGPAT6* GENE POLYMORPHISM WITH MILK PRODUCTION TRAITS AND ITS EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF POSTPARTUM *DEONI* CATTLE

Sapna Nath[1], Dayal Nitai Das[2*], Sukanta Mondal[3], Satish Kumar Illa[4], Kumar Nagaleekar[5], Shiv kumar Tripathi[6]

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ABSTRACT: ***tAGPAT6* gene, which encodes for the enzyme 1-acylglycerol-3-phosphate O-acyltransferase 6, is a vital component in lipid metabolism and plays a significant role in milk fat production in dairy animals. The present study investigated the association of genetic variation within 5′UTR exons 1-1511, 3′UTR exons 1512-1723 of *AGPAT6* gene in *Deoni* cows with milk production traits using PCR-SSCP method. The present investigation revealed three unique SSCP patterns, '1', '2' and '3' in Exon 1 of the *AGPAT6* gene. A total of two novel single nucleotide polymorphisms (SNPs) were identified in the coding region of Exon 1. Both the SNPs were found to cause silent mutation having significant ($p \leq 0.01$) association with fat percentage. The present study further aimed to investigate the relative abundance (%) of mRNA of AGPAT6 gene in PBMC of *Deoni* cows from the day of calving (0th day) to the 60th day post-partum using qRT-PCR. Expression levels of *AGPAT6* were significantly elevated ($p < .05$) on the 30th, 40th, and 60th days postpartum relative to calving day, highlighting its pivotal role in extensive milk fat synthesis during lactation. The variation in expression, with a greater than 10-fold increase from day 0 to day 60, was predominantly driven by the lactation stage. Therefore, enhancing *AGPAT6* gene expression in bloodstream signifies a valuable approach for the early identification of cows exhibiting increased milk fat levels and production during lactation. Additionally, the recognized polymorphisms could serve as genetic indicators for selective breeding.**

Keywords: Polymorphism; Milk production trait; SNP Association; AGPAT6 gene expression.

INTRODUCTION

India leads globally in milk production with 239.3 million tonnes annually, comprising 25% of the total global milk production, of which indigenous cattle contribute approximately 44.76% [1,2]. Among the total 53 cattle breeds in India, *Deoni,* is a prominent dual-purpose breed in western and southern regions. It is recognized for moderate milk production, strong disease resistance, adaptability to hot tropical climates, efficient feed conversion, and cost-effective maintenance [3]. *Deoni* cattle are employed in natural cattle farming to uplift impoverished farming communities [4].

The *AGPAT6* gene on bovine chromosome 27, composed of 12 coding exons, is pivotal in catalyzing glycerol lipid biosynthesis and regulating milk fat synthesis in the bovine mammary gland [5, 6]. Most cells utilize the glycerol phosphate pathway for TAG biosynthesis, involving sequential addition of activated fatty acyl groups to glycerol 3-phosphate [7, 8]. Glycerol-3-phosphate acyltransferases (GPAT) catalyze the initial and rate-limiting step in this pathway by adding fatty acyl groups to the sn-1 position of glycerol 3-phosphate [9, 10]. *AGPAT6* seems to be the predominant AGPAT variant, with its expression

[1]*Assistant Professor (CTF), Department of AGB,* [4] *Scientist and Head, LRS, CVSc Garividi, Vizianagaram, Andhra Pradesh, India.*
[2]*Principal Scientist, Genetics Lab., Dairy Production Section, ICAR- National Dairy Research Institute, Southern Regional Station (SRS), Bengaluru, India.*
[3]*Principal Scientist,* [6]*Ph.D. Scholar, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India.*
[5]*Assistant Professor, Department of AGB, CVSc, Bidar, Karnataka, India.*
**Corresponding author. e-mail: dndasndri@gmail.com*

significantly increased during lactation in the bovine mammary tissue [5, 11]. Deletion of the *AGPAT6* gene in mice results in lactation impairments, characterized by a reduction of approximately 90% in diacylglycerols and triacylglycerols in the milk of double knockout animals [12]. The genetic variation of bovine *AGPAT6* has been systematically examined in both dairy cattle and buffaloes [11,13,14,15].

These findings underscore the essential role of *AGPAT6* in regulating milk fat composition and milk production in dairy cattle. Therefore, the current study sought to identify single nucleotide polymorphisms (SNPs) within the *AGPAT6* gene and analyze their association with milk production traits in *Deoni* cattle using PCR-SSCP and DNA sequencing. Further, considering the significant role of *AGPAT6* gene in regulation of milk fat synthesis, expression pattern of *AGPAT6* gene in post-partum cows during lactation was analysed from peripheral blood mononuclear cells (PBMCs) using qRT-PCR.

MATERIALS AND METHODS

Experimental animals and their management

All the experimental procedures were c onducted as per guidelines of the Institutional Animal Ethics Committee. A total of 100 *Deoni* cows maintained under semi-intensive system of management at Southern Campus of ICAR- National Dairy Research Institute, Bangalore, Karnataka, India were used for the present study. Data on the productive and reproductive performance of *Deoni* cattle were collected from history sheets and breeding cards maintained at the cattle yard. These records were used to estimate both production and reproduction performance, including milk yield, fat percentage, first lactation milk yield, age at first calving, and first service period. Additionally, the data enabled the analysis of genetic parameters, the calculation of breeding values for first lactation milk yield, and the examination of associations between genetic variants of the AGAPT6 gene and performance metrics. To ensure a normal distribution, outliers were excluded, and only data within the range of mean ± 2 standard deviations were included. For genetic studies, only sires with three or more daughters were included. All animals were resided in a spacious housing arrangement within an open enclosure (loose housing system) equipped with covered feed troughs, supplied with high-quality forage and concentrates according to the NRC (2001) standards. Breeding was accomplished using artificial insemination (A.I.), cows were hand-milked two times daily, and thorough veterinary care along with hygiene practices were implemented throughout the year to ensure optimal health of the animals. All creatures were kept under standard management practices following the Minimum Standard Protocols as specified by the Department of Dairying, Animal Husbandry & Fisheries, Government of India.

Milk sampling and phenotype measurement

All cows were milked twice daily and the daily milk yield in kilograms wet weight was recorded by using Lacto Star apparatus (Funke-Gerber, Germany). About 30 ml of milk samples were collected to estimate the fat percentage by Gerber's method. For calibration of Lacto Star apparatus, fat percentage of milk was tested by Gerber method [16].

Blood collection and isolation of DNA

About ten millilitres of venous blood from non-consanguineous individuals was collected aseptically from the jugular vein in a vacutainer tube containing 0.5% ethylene diamine tetra acetic acid (EDTA). Subsequently, the samples were transported to the laboratory under cold conditions and stored at 4°C. Genomic DNA was extracted from the blood within 24 hours of collection by high salt method [17]. The working solution of DNA was prepared by diluting the stock to 100 ng/µL for utilizing as DNA template in polymerase chain reaction (PCR).

Primer design and polymerase chain reaction amplification of AGPAT6 gene

To screen polymorphism in AGPAT6 gene of *Deoni* cows, ten sets of oligonucleotide primers were designed (Table 1) from *Bos taurus* AGPAT6 gene sequence AC_000184.1 using NCBI primer BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and were procured from Xcelris Lab Ltd., Bengaluru, India. PCR was performed in a reaction volume of 25 µl containing approximately 100 ng of genomic DNA, 20 pM of each primer, 200 µM of dNTPs (Bioron GmBH, Germany), one unit of Taq DNA polymerase (Bangalore Genei, India) and 2.5 µl of 10X Pol assay reaction buffer, containing 1.5 mM $MgCl_2$ and supplied with the enzyme buffer. In the process of PCR amplification the annealing temperature (T_a) was optimised for each primer sequence. The thermocycler (Bio-Rad, USA) conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s with varying annealing temperatures based on primer set (Table 1), extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The

PCR amplification was verified by electrophoresis of the PCR products (5 µl) at 100 V in 1.5% (w/v) agarose gel using 1 µl of loading dye (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol) as a stop dye, electrophoresed in 1X Tris Borate EDTA (TBE) (Sigma Aldrich, USA) buffer containing 0.5 µg/mL ethidium bromide (Sigma Aldrich, USA) along with a 100 bp DNA ladder (Sigma Aldrich, USA) as a marker for confirmation of the length of the PCR products. The gels were visualized and documented using UV light in Gel documentation system after ethidium bromide staining (Gel doc 1000, BioRad, Hercules, CA, USA).

Single-strand conformation analysis and sequencing

Amplified PCR products (10 µL) were resolved by running in 10% native PAGE gel [10% acry1amide: bis-acrylamide (29:l, Sigma Aldrich, USA)] gel by using 10 µL denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA Sigma Aldrich, USA) and heat denatured at 95°C for ten minutes. After denaturation PCR products were immediately transferred to chilled ice pack and kept in -20°C for 10 minutes. The vertical gel electrophoresis was carried out in a Bio-Rad Protean® II Xi Cell electrophoreses unit using 1X TBE (Sigma Aldrich, USA) buffer at 200 V for 12 h at 4°C. Gels were silver-stained [18] and photographed using a digital camera for SSCP pattern analysis. Band patterns were characterized by the number of bands and mobility shifts, and each pattern was scored manually for SSCP variants recording. DNA samples showing different patterns on SSCP gels were selected and sent to Eurofins Pvt. Ltd., Bengaluru for sequencing in both directions. Two representative DNA samples for each SSCP variant were subjected to custom sequencing using automated ABI DNA Sequencer 3730 XL (Thermo Fisher Scientific, Meridian Road, Rockford, IL, USA) for detection of single nucleotide polymorphisms (SNPs) using both forward and reverse primers. Multiple sequence alignments were performed with DNA Baser (Heracle BioSof SRL, Pitesti, Romania) and Clustal W multiple sequence alignment software (developed by Des Higgins) for detecting SNPs [19] by comparing the observed sequence of the AGPAT6 gene in Murrah buffaloes with the NCBI Resource Coordinators (2016). Differences between PCR products, as well as between PCR products and reference sequences available in the GeneBank, were classified as experimental and computational SNPs, respectively.

Statistical analysis

Performance data pertaining to 100 *Deoni* cows was collected and classified according to different herds, years and seasons. Effects of different non-genetic factors like season (November-February; winter, March-June; summer, July-October; rainy), period (2002-2012, divided into 5 classes), age at first calving (months, ≤30 to >45-55, divided into 4 classes) and influence of different management practices in the farm and field conditions were included in the analysis. Statistical analyses were carried out using SPSS 22 statistical software (IBM SPSS Inc., Chicago, IL, USA). To ensure normal distribution the outliers were removed by plotting and only data within the range of mean ± 2 SD were considered. For genetic studies, total 7 sires with three or more daughters were considered. The effect of non genetic factors was adjusted by least squares analysis (LSA) implemented in SAS software (Version 9.2). The models were used with the assumption that different component being fitted into the model were linear, independent and additive. The following linear model were used for milk yield and composition traits, respectively.

$$Y_{ijlmn} = \mu + S_i + POC_j + SOC_l + AFC_m + G_n + e_{ijlmn}$$

Where, Y_{ijlmn} = observed value of milk yield at j^{th} period of calving, at l^{th} season of calving and m^{th} group of AFC, G_n = fixed effect of n^{th} SSCP pattern set of twelve exons with µ = overall mean, Si = ith sire (i = 1,2,3, . .7), POC = j^{th} period of calving (l= 1, 2, 3, 4and 5), SOC = l^{th} season of calving (November-February (winter 1), March-June (summer) 2, July-October (rainy) 3), AFC = m^{th} age at first calving (n = 1, 2, 3,4), e_{ijklmn} = random error which is normally and independently distributed with mean 0 and unit variance σ^2_{e} and

$$Y_{ijklmn} = \mu + a_i + b_j + c_k + d_l + f_m + e_{ijklmn},$$

where: Y_{ijklmn} = observed value of milk composition trait; m= trait mean; a_i= genotype effect (i= 1,2); b_j=lactation number effect (j = 1,.,4); c_k = calving age effect (k = 1,.,4); d_l = calving season effect (k = 1,.,4), f_m = lactation month effect (m = 1,.,4); e_{ijklmn} = error.

Methodology for AGPAT6 gene expression in peripheral blood mononuclear cells of post-partum *Deoni* cows

Total nine *Deoni* cows with milk yield averaged (884.5kg/ lactation), in 2nd &3rd lactation, with 4.09 ± 0.10 % fat in milk/lactation, were selected from LRC at ICAR- NDRI, SRS, Bangalore, India. 15 mL of blood from each animal was collected from external jugular vein into an EDTA vacutainer at a regular interval

of 10 days from the day of parturition up to 60th day post- partum. Fat content in test day milk samples was estimated by employing Gerber's method [16]. Milk samples was collected from cows at two lactation stages: the early stage and peak stage, at a regular interval of 10 days from the day of parturition up to 60th day post- partum.

PBMC preparation and plasma separation of whole blood

Fresh blood samples were collected in an EDTA vacutainer and were processed immediately for PBMC isolation as per the standard protocol. The whole blood (15 ml) was carefully layered over the PBMC separation medium (Histopaque-1077) (Sigma, USA), kept in a 50-ml conical centrifuge tube (Fig. 5) and centrifuged for 30 min @ 1000 × g. The resulted PBMC pellets were maintained at −80°C for further analysis. The blood samples were immediately processed on the same day for RNA isolation from Peripheral Blood Mononuclear Cells (PBMC) and cDNA synthesis.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from the PBMC pellet using RNeasy Minikit (Qiagen, Netherlands) as per the manufacturer's instruction. Purity of the extracted RNA was determined by evaluating at A260/A280. Total RNA obtained was reverse-transcribed using Super Script III First Strand Synthesis system (Invitrogen, USA). Quality and quantity of cDNA were checked by the Nanodrop spectrophotometer (Eppendorf, Germany), and the resulted cDNA templates were used for PCR amplification. Transcript abundance for AGPAT6 gene using cDNA from PBMC of post-partum dairy cows was compared with three replicates per sample using quantitative real-time PCR (qRT-PCR). Real-time PCR analysis was performed using the ABI Step One Plus real-time cycler (Applied Biosystems, USA), Fast SYBR Green Master Mix (Applied Biosystems, USA) and gene-specific primers for both housekeeping and target genes (Table 2).

For each sample, the PCR consisted of 2 µl of cDNA, 10 µM of each primer and 10 µl of Fast SYBR Green Master Mix (2×) in a final volume of 25 µl per reaction. Reaction conditions included an initial step of 95°C (20 s), followed by 40 cycles of 95°C (30 s) and 60°C (25 s) and a melt curve of 95°C (15 s), 60°C (1 min) and 95°C (15 s) followed by use of melt curve analysis. A single sharp peak in the melt curve analysis and single band in gel electrophoresis indicated specific amplification for primer pair. No template control was also included for the primer assay. Two biological replicates and three technical replicates were used for each sample. qRT-PCR data (CT values) were analysed using the $2^{-\Delta\Delta ct}$ method [20]. The specificity of each PCR product was determined by amplicon size determination in 2.0 % agarose gel electrophoresis (Fig. 6).

Data normalization and analysis

In the present investigation, the Norm Finder algorithm was used. The housekeeping gene β-actin was used as reference index and used for normalization (Fig.7 and Fig. 8). Quantitative cycle (Ct) values were calculated by Applied Biosystems 7500 software v2.0.5. Relative mRNA levels were then calculated for each gene using ΔΔCt method. Ct of housekeeping genes were subtracted from Ct of each gene to obtain ΔCt. Calibrator ΔCt was subtracted from each sample's ΔCt, and then relative mRNA value was calculated by the formula $2^{-\Delta\Delta ct}$ [20] (Fig. 9 and Fig. 10).

Statistical analysis

Statistically significant differences ($p<0.05$) in relative AGPAT6 gene expression levels were determined using the one-way analysis of variance (ANOVA) of SAS 9.3 (SAS Institute Inc.) software package followed by the Post-hoc multiple comparisons was made by Tukey's multiple range test.

RESULTS AND DISCUSSION

Milk production and fat yield

Milk production was found to be 2.96±0.71, 2.83±0.74, 2.39±0.75, 2.2±0.77, 2.07± 0.79, 1.82 ± 0.817 and 1.61 ± 0.785 Kg/day/milking, respectively, on the day of calving (0th day), 10th, 20th, 30th, 40th, 50th and 60th day post partum. In addition, milk fat percentage was found to be 5.96±0.15, 5.65±0.16, 5.26±0.16, 4.63±0.075, 4.42±0.065, 4.25±0.094 and 4.02±0.102 % of milk fat/ day, respectively, on the day of calving (0th day), 10th, 20th, 30th, 40th, 50th and 60th day post partum. *Deoni* cows have a higher level of fat percentage as compared to the crossbreds [21]. Before milking, every time calves are left to suckle their mothers for easy let down of milk.

Molecular characterization of *AGPAT6* gene

A 2,295-bp PCR product of the *AGPAT6* gene, encompassing twelve exons, flanking introns, and untranslated regions, was amplified. Polymorphism within the AGPAT6 gene was examined using ten sets of overlapping primers through PCR-SSCP analysis.

Table 1. Details of primer sequences [5'to 3' sequences] used for amplification.

Exon	Sequence	Amplicon length (bp)	Annealing Temperature (°C)	SSCP condition
Exon 1	F- CGAACTTGGGAATGGAGTTG R- GTCCCTCTGAGAGCTCACAATC	384bp	54.3	18^0C, 80V, 10h
Exon 2	F- CTGTCACAGACTGGAAGTGGAG R- GCAACAGAACAGTCTCATCAGG	275bp	55.8	18^0C, 80V, 8h
Exon 3	F-CCTGATGAGACTGTTCTGTTGC R- ACCACCCTCCTCTGAAGTGAG	372bp	55.5	18^0C, 80V, 10h
Exon 4	F- ACAGGATAGCTCTCGCTTTCAC R- ATAAGATCAGTGAGGGGGAACA	221bp	54	18^0C, 80V, 10h
Exons 5 and 6	F- GTGCGTGTAGGTAACAGCAGAG R- CAGGTCTGAAAGCACACTTGC	449bp	58	18^0C, 80V, 12h
Exon7	F- TGGTTACCAGTAAAGCGTAGGG R- CGACTTGCCCTAAAATCAGACT	256bp	54	18^0C, 80V, 8h
Exon8	F- ACAGATGTATGGATGGTGATGG R- ACTCGCAGTCCACACTGAGTC	263bp	55	18^0C, 80V, 8h
Exon9	F- AAGAGCTGTCAGAGGGCTTTTA R- AGAGGAACACGTGGGGAGAT	304bp	53.4	18^0C, 80V, 10h
Exons 1 and 11	F- GCACACACTGACACACACTCTT R- AGCAACCGCCTCAGAAATC	419bp	52.9	18^0C, 80V 12h
Exon 12	F- TGCCATGCTCTTGTCAGG R- CACACGGGCATCAGACAG	338bp	51.5	18^0C, 80V, 10h

Table 2. Details of primers used RT-PCR.

Primer Name	Primer Sequence [5' to 3']	Length[bp]	Annealing Temperature [°C]
AGPAT6 Forward	CGAGTTTGAGCTCTCGGATATT	250bp	55
AGPAT6 Reverse	AGCGAGAGCTATCCTCAGTGAC		
β Actin Forward	GGCATCCTGACCCTCAAGTA	207bp	55
β Actin Reverse	CACACGGAGCTCGTTGTAGA		

Table 3. Genotype frequencies of Exon1 of *AGPAT6* gene based on SSCP pattern in Deoni cattle.

Region	Pattern/genotype	Number of observations	Frequency of SSCP variants	Chi square value
Exon 1	Pattern 1	32	0.32	0.416
	Pattern 2	37	0.37	
	Pattern3	31	0.31	

Analysis of PCR-SSCP amplicons from exon 1 revealed polymorphism, whereas the other exons were found to be monomorphic. Exon 1 exhibited polymorphism within the population, manifesting in three distinct SSCP patterns with varying mobility shifts (Fig. 1). The frequencies of SSCP variants patterns 1, 2, and 3 in exon 1 were determined to be 0.32, 0.37, and 0.31, respectively. Chi-square analysis revealed that the screened population is under Hardy Weinberg equilibrium (χ^2=0.41 <5.99) (Table. 3). The purified PCR products were submitted for custom sequencing at Eurofins Pvt. Ltd., Bengaluru, and analyzed. To identify the QTNs in the *AGPAT6* gene, the reference sequence of *Bos taurus* (AC_000184.1) was aligned with the edited sequences of *Deoni* cattle using Clustal W software, revealing two nucleotide variations in Exon 1 of the *AGPAT6* gene. Insertions of guanine (G) at positions 915 and 916, and thymine (T) at positions 920 and 921 were identified in exon 1. These insertions did not lead to any amino acid changes in the observed SSCP variants (patterns 1, 2, and 3) (Fig. 2 and 3).

Table 4. Association of the AGPAT6 gene exon 1 patterns with milk production traits of Deoni cattle given as least square means with standard error (LSM± SE).

AGPAT 6 Exon 1 Patterns	First lactation milk yield	Average fat percentage
Pattern 1**(32)	490.36±67.84	4.14 [a] ±0.11
Pattern 2*(37)	572.35±66.39	3.15 [b] ±0.11
Pattern 3**(31)	616.80±73.26	5.16 [a] ±0.12
p-Value	0.780 (NS)	0.000(**)
Combined effect of patterns		
P1+P2	542.06 [a] ±48.26	4.11 [b] ±0.09
P2+P3	604.57 [ab] ±48.46	4.17 [b] ±0.10
P1+P3+P2	565.24 ±49.02	4.15 ±0.10
p-Value	0.327[NS]	0.004**

*Values bearing different superscripts between rows differ significantly from each other and P values bearing * and ** between rows differ significantly at p≤0.05 and p≤0.01 level. P1, pattern 1; P2, pattern 2 and P3, pattern 3 of exon 1. Values bearing NS shows non significance.Pattern sets having frequency less than 10 were removed from analysis.

Table 5. Least squares analysis of variance for non-genetic effects on 305 days milk yield and average fat percentage. [Comparison among the least-squares means of 305 days milk yield and average fat percentage for the non-genetic effects with different patterns of AGPAT6 gene].

Trait	Source	DF	Mean squares
305 days milk yield	Sire	6	18326.49
	Period	4	4676.19
	Season	2	1075.86
	AFC	3	17088.39
	Error	84	10142.67
Average fat percentage	Sire	6	128209.65*
	Period	4	469507.70**
	Season	2	27688.78
	AFC	3	99501.02
	Error	84	119868.69

The sequences obtained were analyzed using BLAST to determine their similarity with sequences from other species. The analysis of the entire coding sequence showed homologies of 97%, 97%, 97%, 98%, 98%, and 59% with *Bos indicus* (XM_019953472.1), *Bison bison bison* (XM_010846369.1), *Bos Taurus* (NM_001083669.1), *Bubalus bubalis* (NM_001290846.1), *Sus scrofa* (NM_001145019.1) and *Ovis aries* (XM_004021798.3), respectively (Fig. 4). It was observed that there was a consistent trend of increasing genetic distance, varying in degree, between the Deoni breed and other livestock species such as *Bos taurus, Bos indicus, Bison bison bison, Capra hircus, Ovis aries, Bubalus bubalis,* and *Sus scrofa*. Analysis of all exons of AGPAT6 indicated genetic distance differences across species.

Association of genetic variants with milk yield and fat content

In Deoni cattle the overall first lactation milk yield (Kg) obtained was 561.88 ± 37.5 and fat percent (%) was found to be 4.15 ± 0.13(Table 4). The association study indicated a significant ($p < 0.05$) effect of SSCP variants in exon 1 on milk fat, however, these variants did not significantly affect milk yield. The calving period was found to have a significant effect ($p < 0.05$) on average fat percentage in Deoni cattle. In the present study, neither the season of calving nor the age group at first calving significantly affected average fat percentage. Similarly, age at first calving, parity, and season did not significantly impact milk yield (Table 5). Differences in average fat percentage in Deoni cattle may be attributed to variations in fodder availability, feeding practices, management strategies, and environmental conditions such as precipitation, humidity, and temperature during different periods. Table 4 presents the association of SSCP patterns in exon 1 with milk production traits. Among the three SSCP variants of exon 1, cows with patterns 1 and 3 exhibited significantly higher fat percentage.

SSCP variants of pattern 1 + pattern 2 of exon 1 had significant ($p<0.05$) effect on milk production whereas, the combined effect of all the patterns was found to have a highly significant effect on average fat percentage ($p<0.01$). Cows with pattern 2 +pattern 3 was found to have the highest 305 days milk yield (604.57±48.46) and highest average fat percentage (4.17±0.10) compared to combined pattern 1 +pattern 2 +pattern 3 of exon 1 (565.24±49.02and 4.15±0.10) (Table 4).

Table 6. AGPAT6 gene expression on different days of post-partum in Deoni cows.

Expression of AGPAT6 mRNA	Post-partum (Mean±SE)	Controls(Mean±SE)	p value
Day 0 (day of calving)	0.401±0.32	0.01±0.10	0.18
Day10	0.409 ± 0.06	0.23±0.01	0.07
Day 20	0.608 ±0.26	0.21±0.13	0.09
Day 30	0.653±0.37	0.19±0.11	0.042*
Day 40	1.477±0.76	0.17±0.12	0.017**
Day 50	1.351±1.38	0.14±0.07	0.012**
Day 60	2.065±1.02	0.11±0.05	0.008**

* p values bearing * and ** between rows differ significantly at p≤0.05 and p≤0.01 level.

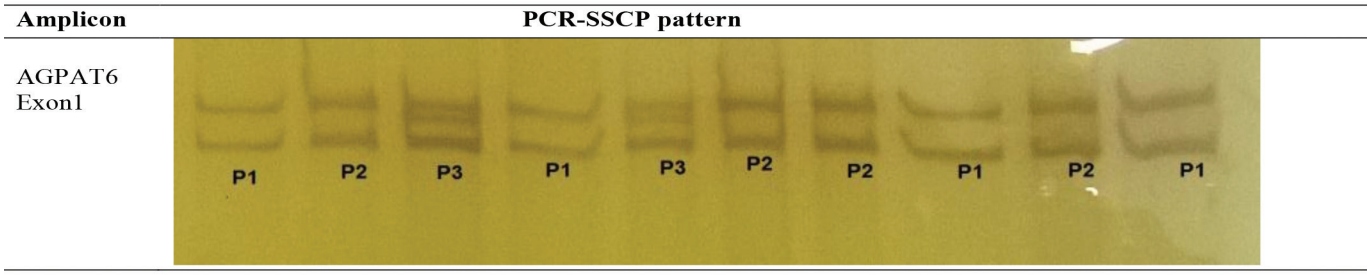

Fig. 1. PCR- SSCP pattern of AGPAT6 gene in Deoni cattle.

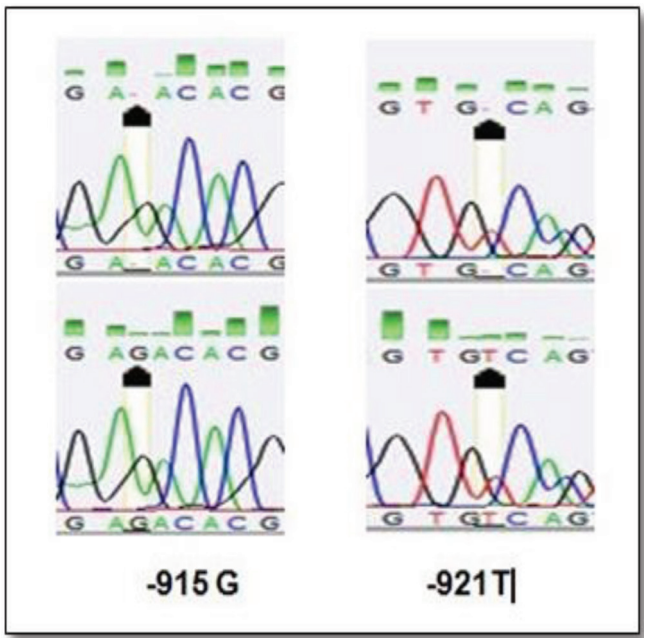

Fig. 2. Sanger trace figures of SSCP variant sites of AGPAT6 exon 1 in Deoni cattle [insertion at position 915G and 921T].

PBMC separation and RNA isolation from whole blood

Amplification of partial *AGPAT6* cDNA sequence of 250 bp from *Bos indicus* in different days of post-partum was amplified (Fig. 5) which corresponds to the *Bos taurus AGPAT6* mRNA sequence (Acc. No. NM_001083669.1).

Fresh blood samples collected on different days *viz.* on the day of calving (0th day), 10th, 20th, 30th, 40th, 50th and 60th day post partum were processed immediately for isolation of peripheral blood mononuclear cells. Purity of total RNA isolated from the PBMCs was in the range of 1.9 to 2.34, and the average concentration of RNA obtained was 367 ng/µl.

***AGPAT6* mRNA expression in post-partum dairy cows**

AGPAT6 mRNA expression in PBMCs of post-partum dairy animals was monitored every 10 days from calving (control) to the 60th day post-calving. In Deoni cows, *AGPAT6* gene expression showed an upward trend from 0th day to 60th day post-partum. Specifically, *AGPAT6* levels increased from 30th day after parturition, peaked on 40th day, and remained elevated until 60th day (Figure 11). In post-partum cows from calving to the 30th day post-calving, there was no significant increase observed in *AGPAT6* expression levels. This suggests that *AGPAT6* expression is higher during lactation compared to the period immediately after parturition, indicating higher fat levels during lactation in Deoni cows. The expression of *AGPAT6* was notably affected by the stage of lactation, as indicated in Table 6, with a greater than 10-fold difference observed between day 0 and day 60.

Comprehending the genetic underpinnings of milk production traits, can facilitate the formulation of

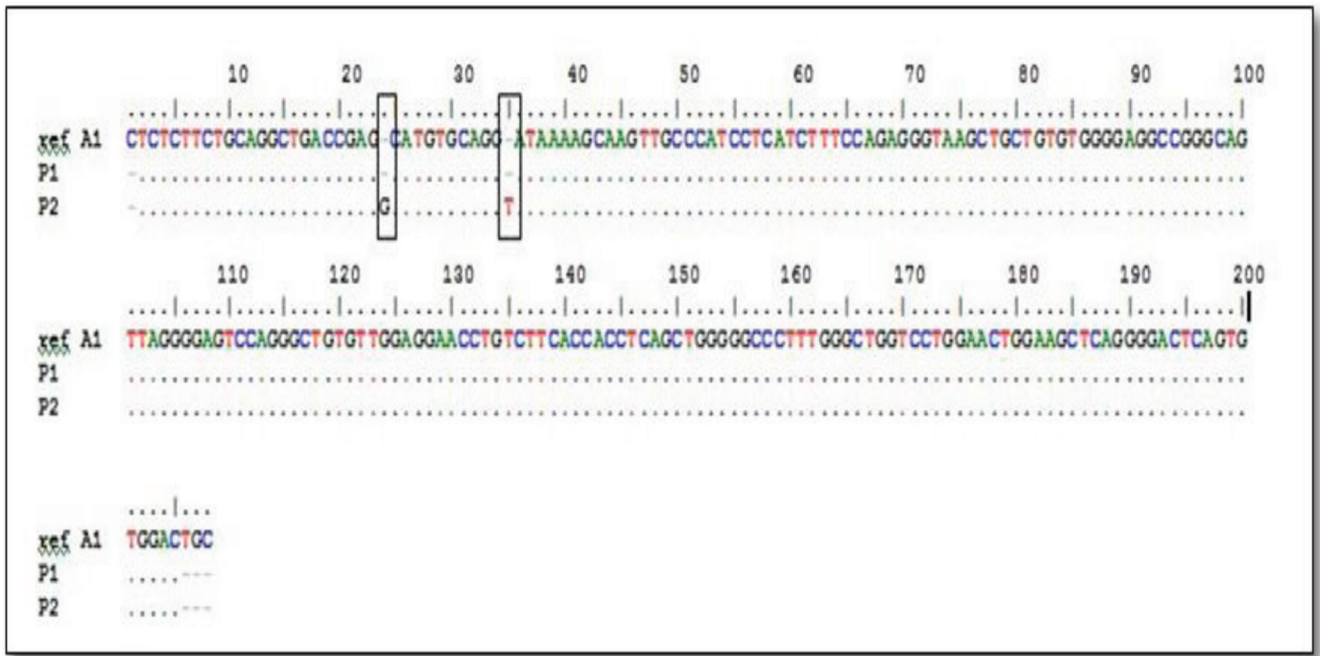

Fig. 3. Clustal W multiple sequence alignment of AGPAT6 Exon 1 in Deoni cattle.

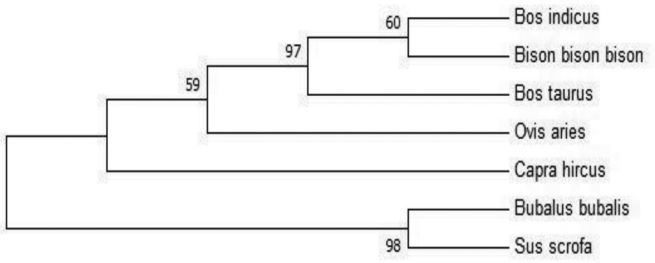

Fig. 4. Neighbor-Joining dendrogram showing the relationships of AGPAT6 gene among some species. (Percent bootstrap values, obtained from 1000 replications of resampling factors, are shown for each node).

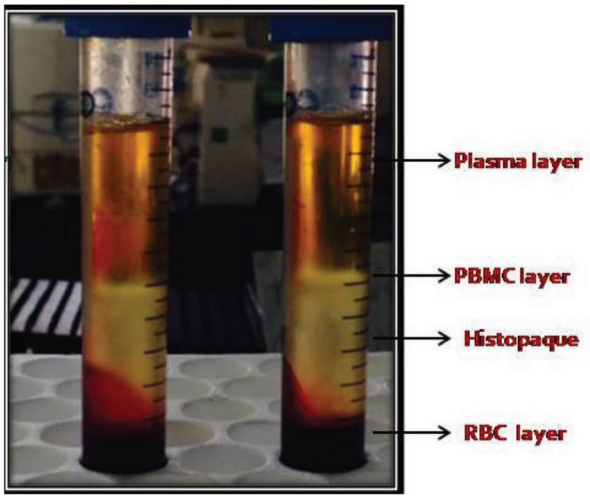

Fig. 5. PBMC separation from whole blood.

more effective breeding strategies for dairy animals. The identification of genetic variations through SNPs and their strong association with economically important traits are considered potent tools for guiding marker-assisted selection [22]. The discovery of genetic variations within the AGPAT6 gene and their correlations with milk fat traits are crucial factors to consider in milk production and animal breeding strategies [23, 24, 25].

The current study identified two SNPs in the AGPAT6 gene in Deoni cattle. Our characterization of the AGPAT6 gene aligns with previous research by [11], who documented ten variants involving amino acid substitutions across 5'UTR exons 1 and 2, and the connecting intron in the AGPAT6 gene in *Bos taurus* cows. Present investigation discovered that cows exhibiting patterns 2 and 3 in exon 1 of the AGPAT6 gene could serve as markers for selecting dairy cows with high fat percentages. Moreover, both coding and non-coding regions of the Exon 1 of AGPAT6 gene displayed high polymorphism and were strongly associated with milk production traits in Deoni cattle.

HAYR (2014) [26], identified additive and dominance effects on the AGPAT6 gene in 4,801 Holstein Friesian cows using BayesB in GenSel with 35,000 SNPs. They found that AGPAT6 heterozygotes had a distinct mean milk fat yield compared to QQ

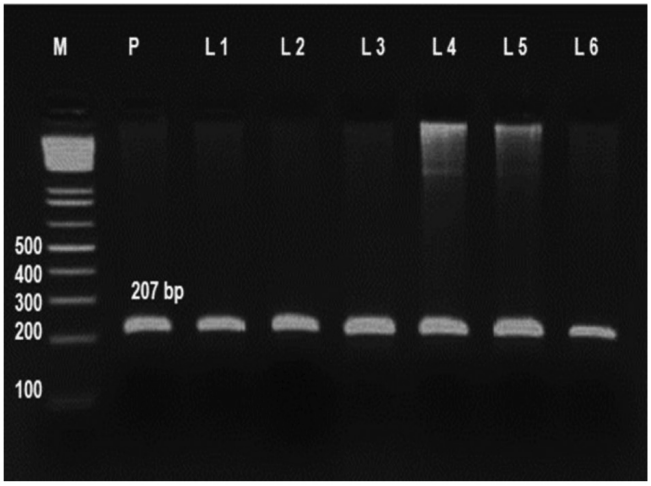

Fig. 6. Agarose gel electrophoresis (2%) of Real time PCR showing amplified AGPAT6 gene product from cDNA.

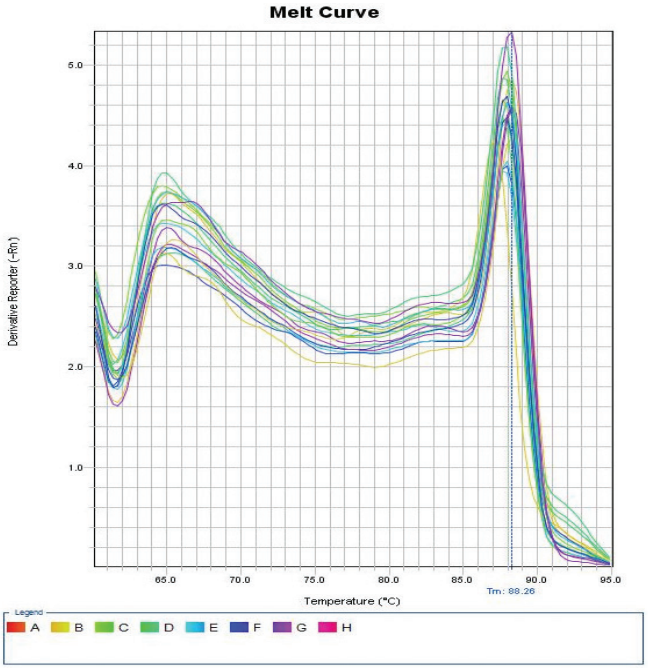

Fig. 8. Dissociation curve for AGPAT6 primer at 200ng of cDNA Concentration.

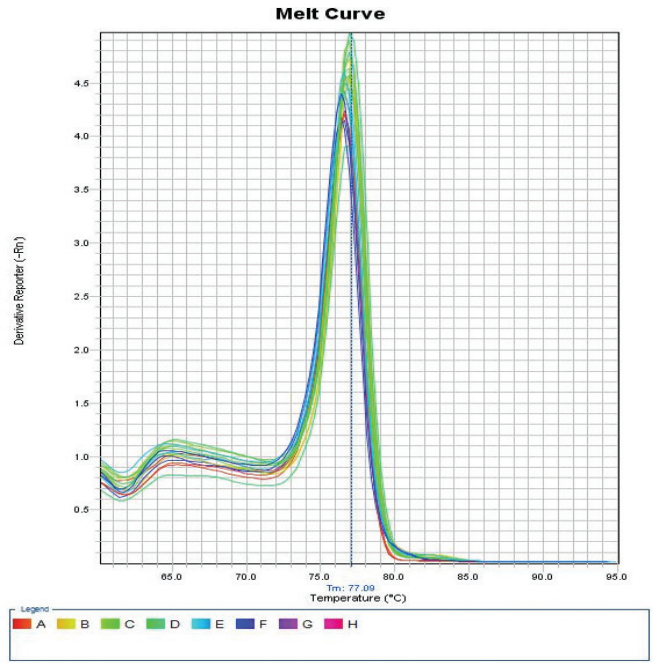

Fig. 7. Dissociation curve for β actin primer at 200ng of cDNA Concentration.

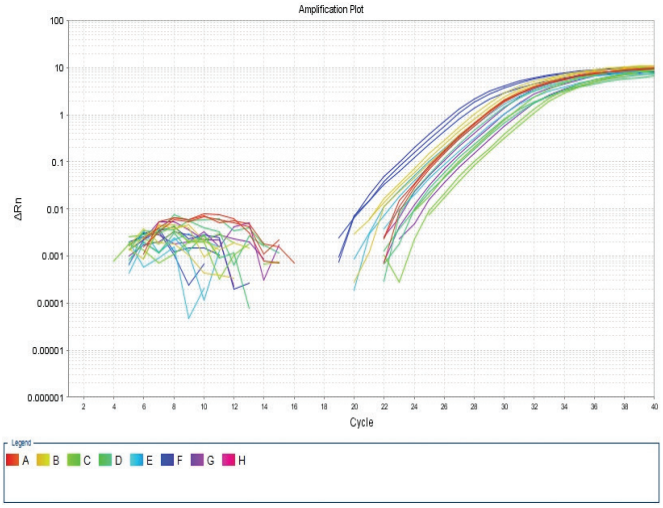

Fig. 9. Amplification plot for β actin gene in RT- PCR.

homozygotes (p=0.03). SONG *et al.* (2013) [13] sequenced the full coding sequence (CDS) of the AGPAT6 gene using RT-PCR in 12 water buffalo. Further, [13] analyzed AGPAT6 gene expression variants in biopsies from lactating mammary tissue of Holstein Friesian and Holstein Friesian-Jersey crossbred cows. They found that certain genotypes led to an increase in mammary AGPAT6 gene expression during mid-lactation, which correlated with elevated milk triglyceride synthesis. Present findings are in harmony with other researchers [14] were they have reported significant association of AGPAT6 gene polymorphism with milk fat percentage with estimated effects ranging from 0.090 to 0.113% ($p < 0.01$).

This study presents the initial evidence of AGPAT6 gene polymorphism identified through PCR-SSCP, influencing milk yield and fat content in Deoni cattle. Among the three SSCP variants of exon 1, cows with pattern 3 had higher milk yield whereas, cows with pattern 1 and 3 were found to have high fat percentage as compare to cows with pattern 2. The sequence examination of each SSCP variant among

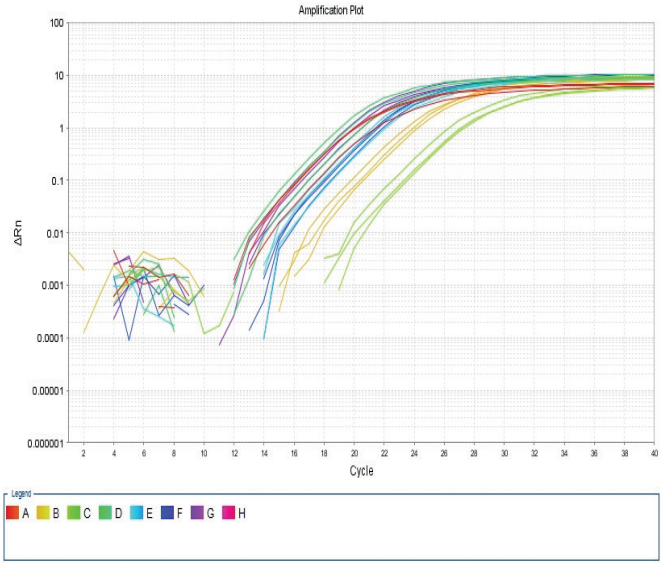

Fig. 10. Amplification plot for AGPAT6 gene in RT-PCR.

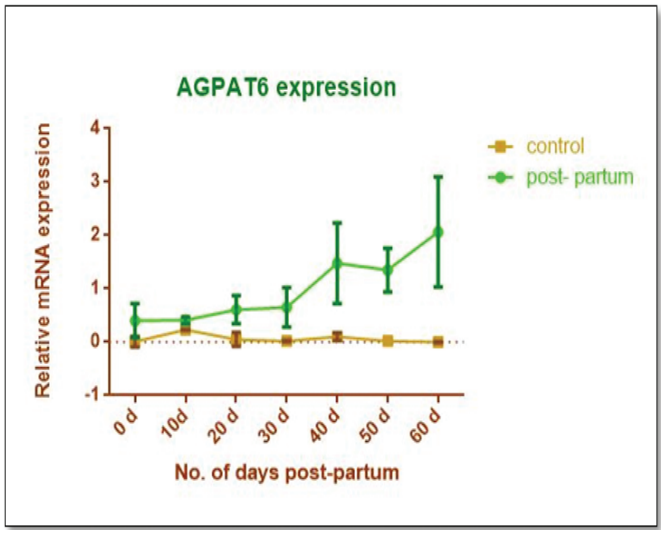

Fig. 11. Relative expression of AGPAT6 mRNA from 0th day to 60th day post-partum in Deoni cows.

the investigated Deoni cow population uncovered two SNPs in the AGPAT6 gene. Both were insertions (G at 915_916 and C at 920_921) in exon 1, manifested in SSCP variant as three distinct patterns. Although classified as a silent mutation, recent studies have suggested a link between silent mutations and gene functionality [27]. Notably, proteins that share identical amino acid sequences but possess differing gene sequences can display diverse structural and functional characteristics [28]. Grasping the mechanisms underlying silent mutations could yield significant insights. Since variations in AGPAT6 expression predominantly influence fat synthesis, these pleiotropic effects are likely channelled through secondary mechanisms that modulate milk composition [29, 30].

The *AGPAT6* gene exhibits widespread mRNA expression across various tissues [13,15]. The current investigation further elucidates the expression profile of AGPAT6, a pivotal gene in milk lipogenesis, in the Deoni cattle breed through 60 days postpartum. The *AGPAT6* gene showed varying expression levels in post-partum dairy cows, with higher expression observed on the 60th day compared to the 10th day post-partum. Our results concerning the expression of the *AGPAT6* gene corroborated those of previous studies [31]. They reported a 15-fold up-regulation in *AGPAT6* mRNA abundance during peak lactation (60th day post-partum) compared to early lactation stages (15 days ante partum), followed by a decline in late lactation in Holstein-Friesian cattle. [11] documented that increase AGPAT6 gene expression correlates additively with a high milk fat percentage genotype in Bos Taurus cows. This suggests the significant role the *AGPAT6* gene may play in the synthesis of milk fat in *Bos indicus* cattle. [5] discovered that the *AGPAT6* gene is the primary isoform present in the bovine mammary gland, with its expression levels intricately linked to the lactation stage. In cattle, *AGPAT6* gene expression varies under different physiological states [32]. The fluctuations in *AGPAT6* expression in Deoni cattle could be influenced by a range of factors, given that the functional aspects of *AGPAT6* at the protein level have not been addressed in this investigation. The *AGPAT6* gene demonstrates diverse biological activities in different tissues and under varying physiological conditions.

CONCLUSION

Based on our findings, the *AGPAT6* gene displays genetic variability in *Bos indicus* cows. The SSCP patterns identified in exon 1, specifically patterns 2 and 3, are significantly correlated with milk fat percentage. Further validation of these SSCP patterns across a large number of Deoni cows from diverse herds is warranted for potential application as genetic markers in Marker Assisted Selection. Furthermore, this preliminary study in indigenous Deoni (*Bos indicus*) cattle demonstrates that AGPAT6 expression in PBMCs correlates with variations in fat content and milk yield. In conclusion, AGPAT6 levels can reliably estimate fat content and milk yield in post-partum cows as early as the 60th day. The decrease in AGPAT6 mRNA expression from 0th day to 30th day post-partum in cows may be attributed to

differential gene expression in mammary epithelial cells (MECs). Therefore, increase AGPAT6 gene expression in peripheral blood presents a promising approach for the early detection of cows with higher milk fat content and yield during lactation.

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